OPTIMISATION OF BIOHYDROGEN PRODUCTION BY LOCALLY ISOLATED *Klebsiella* sp. STRAIN PR2

AMALINA BINTI RAMLI

UNIVERSITI TEKNOLOGI MALAYSIA

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AMALINA BINTI RAMLI

A thesis submitted in fulfilment of the requirements for the award of the degree of Master of Philosophy (Bioscience)

Faculty of Science
Universiti Teknologi Malaysia

AUGUST 2018

Alhamdulillah, this thesis dedicated to UTM, my family and my dearest friends.

ACKNOWLEDGEMENT

In the name of Allah the Most Gracious and Most Merciful.

I am grateful for His permission that has helped me to finish this study. First of all I would like to thank my family, especially my beloved and supportive husband who has given the space and opportunity to me to further my studies.

Secondly, I would like to thank my supervisor Prof. Dr. Zaharah Ibrahim who was constantly advising me from the beginning until the end of research and not forgetting my co-supervisor Assoc. Prof. Dr. Madihah Md Salleh who guided me to analyse the research data.

Finally, to all my friends (including FS staff and students) who helped me directly and indirectly throughout my research process. Thank you very much.

ABSTRACT

Nowadays, 80% of the world energy requirement depends on fossil fuels. This ultimately will lead to a reduction in global fossil fuel resources. Hydrogen is a sustainable, clean source of energy and can be produced through biological process by microorganisms. The biohydrogen production is important due to both environmental and economic factors, since waste substrates can be used to generate biogas rich in biohydrogen. The aim of this study was to isolate biohydrogen producing bacteria from agricultural wastewater and to optimise the biohydrogen production using enrichment medium. Out of 25 bacteria obtained, only 13 isolates had the ability to produce biohydrogen. One isolate that produced the highest biohydrogen was selected for 16S rDNA sequence identification. The bacterium was identified as *Klebsiella* sp. strain PR2. For the optimisation of biohydrogen production, fermentation was carried out in batch culture, where parameters such as carbon and nitrogen source concentration, initial pH of the medium, inoculum size, as well as incubation temperature were investigated. D-mannitol was found to be the best carbon source while peptone was the best nitrogen source for biohydrogen production with concentration of 0.2% (w/v) and 0.5% (w/v), respectively. The optimum condition for biohydrogen production was achieved at the initial pH 8 of the medium, incubation temperature of 30 °C and 10% (v/v) of inoculum. After 24 hours of incubation, the highest yield obtained ($Y_{P/X}$) was 8547.42 g/g while the highest biohydrogen productivity was 177.24 mol/L/h. Biohydrogen production using palm oil mill effluent (POME) as substrate was also conducted. Fermentation was carried out in sterile raw and final discharge POME by inoculating with 10% (v/v) bacteria inoculum and incubated at 30 ^{o}C for 5 days. The maximum biohydrogen productivity from raw and final discharge POME were 1.44 mol/L/h and 1.81 mol/L/h, respectively. The highest colour removal were 38% (initial: 18167 ADMI) for raw discharge POME and 31% (initial: 1873 ADMI) for final discharge POME were obtained after 5 days of treatment. Meanwhile, the highest COD removal were 34.8% (initial: 7320 mg/L) and 50.8% (initial: 1410 mg/L) for the raw and final discharge POME, respectively. From this study, it could be concluded that *Klebsiella* sp. strain PR2 has potential applications for biohydrogen production utilising POME as substrate besides treating the POME through biodegradation process.

ABSTRAK

Pada masa kini, 80% daripada keperluan tenaga dunia bergantung kepada bahan api fosil. Ini akhirnya akan membawa kepada pengurangan sumber bahan api fosil global. Hidrogen adalah sumber tenaga yang lestari, bersih dan boleh dihasilkan melalui proses biologi oleh mikroorganisma. Penghasilan biohidrogen adalah penting disebabkan oleh faktor alam sekitar dan ekonomi, memandangkan substrat sisa boleh digunakan untuk menghasilkan biogas yang kaya dengan biohidrogen. Tujuan kajian ini adalah untuk memencilkan bakteria yang boleh menghasilkan biohidrogen daripada air sisa pertanian dan mengoptimumkan pengeluaran biohidrogen menggunakan media yang diperkaya. Daripada 25 bakteria yang diperoleh, hanya 13 bakteria yang mempunyai keupayaan menghasilkan biohidrogen. Satu bakteria yang menghasilkan biohidrogen tertinggi dipilih untuk dikenalpasti melalui jujukan 16S rDNA. Bakteria tersebut dikenali sebagai *Klebsiella* sp. strain PR2. Bagi pengoptimuman penghasilan biohidrogen, fermentasi dijalankan dalam kultur kelompok, di mana parameter seperti kepekatan sumber karbon dan nitrogen, pH awal medium, saiz inokulum, serta suhu inkubasi telah dikaji. D-manitol dikenalpasti sebagai sumber karbon terbaik manakala pepton adalah sumber nitrogen yang terbaik untuk penghasilan biohidrogen dengan kepekatan 0.2% (b/i) dan 0.5% (b/i), masing- masing. Keadaan optimum untuk penghasilan biohidrogen dicapai pada pH awal medium 8, suhu inkubasi 30 °C dan 10% (i/i) inokulum. Selepas 24 jam inkubasi, hasil biohidrogen tertinggi diperolehi ($Y_{P/X}$) adalah 8547.42 g/g manakala produktiviti tertinggi adalah 177.24 mol/L/h. Penghasilan biohidrogen menggunakan sisa efluen kelapa sawit (POME) sebagai substrat juga dijalankan. Fermentasi telah dilakukan dalam POME mentah dan POME pelepasan akhir yang steril dengan menambah 10% (i/i) inokulum bakteria dan diinkubasi pada 30 ^{o}C selama 5 hari. Produktiviti biohidrogen maksimum dari POME mentah dan POME pelepasan akhir adalah 1.44 mol/L/h dan 1.81 mol/L/h, masing-masing. Penyingkiran warna tertinggi adalah 38% (awal: 18167 ADMI) untuk POME mentah dan 31% (awal: 1873 ADMI) untuk POME pelepasan akhir diperoleh selepas 5 hari rawatan. Sementara itu, penyingkiran COD tertinggi adalah 34.8% (awal: 7320 mg/L) dan 50.8% (awal: 1410 mg/L) untuk POME mentah dan POME pelepasan akhir, masing-masing. Dari kajian ini, dapat disimpulkan bahawa *Klebsiella* sp. strain PR2 berpotensi untuk menghasilkan biohidrogen menggunakan POME sebagai substrat di samping merawat POME melalui proses biodegradasi.

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LIST OF ABBREVIATIONS

pH	-	Hydrogen ion concentration
H_2	-	Hydrogen gas
H^+	-	Hydrogen ion
dw	-	Dry weight
ADMI	-	American Dye Manufacturing Institute
C	-	Carbon
N	-	Nitrogen
$C_6H_{12}O_6$	-	Glucose
H_2O	-	Water
CH_3COOH	-	Acetic acid
CO_2	-	Carbon dioxide
CO	-	Carbon monoxide
NH_4Cl	-	Ammonium chloride
KH_2PO_4	-	Potassium dihydrogen phosphate
$MgCl_2$	-	Magnesium chloride
$CaCl_2$	-	Calcium chloride
NaCl	-	Sodium chloride
K_2HPO_4	-	Potassium hydrogen phosphate
$MgSO_4$	-	Magnesium sulphate
$MnSO_4$	-	Manganese(II) sulfate

$FeSO_4$	-	Iron(II) sulfate
HCl	-	Hydrochloric acid
MoO_3	-	Molybdenum trioxide
$ZnSO_4$	-	Zinc sulfate
$CuSO_4$	-	Copper(II) sulfate
H_3BO_3	-	Boric acid
CoCl	-	Cobalt(II) chloride
NaOH	-	Sodium hydroxide
ppm	-	Parts per million
PEG	-	Polyethylene glycol
H_2SO_4	-	Sulphuric acid
$AgSO_4$	-	Silver(II) sulphate
K_2Cr_2O	-	Potassium dichromate
DNA	-	Deoxyribonucleic acid
PCR	-	Polymerase chain reaction
EDTA	-	Ethylenediaminetetraacetic acid

LIST OF SYMBOLS

°C	-	Degree celcius
h	-	Hour
mL	-	Milliliter
L	-	Liter
%	-	Percent
v/v	-	Volume over volume
w/v	-	Weight over volume
µM	-	Micromolar
rpm	-	Rotation per minute
µL	-	Microliter

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Hydrogen gas is an ideal, clean, sustainable energy resource that is harmless to the environment (Suzuki, 1982). Hydrogen can be produced through electrochemical and thermochemical processes. However, it is more environmentally clean when produced through biological process (Nowotny *et al.*, 2016; Das, 2001). Hydrogen gas is commercially used in industries for chemicals and electronic devices production, steel processing, also hydrogenation of oils and fats in food industries (Kapdan and Kargi, 2006). Furthermore, the use of hydrogen as an energy resource is more profitable if it can be produced naturally or at a low cost. The use of wastewater such as palm oil mill effluent (POME) containing microorganisms is one of the approaches for biohydrogen production.

Biological processes of hydrogen production are normally assisted by microorganisms in water at ambient temperature and pressure. The biohydrogen generation system are usually carried out by different type of microorganisms such as photosynthetic bacteria, fermentative bacteria, green algae and cyanobacteria (Das, 2001). Microorganisms have their own unique mechanisms of biohydrogen production depending on the raw materials used. In addition, the environment condition are also important and should be well suited for microorganisms to catalyse the substrate (Das *et al.*, 2008).

Malaysia is one of the world's largest exporter and producer of palm oil and its products, due to rapid development over the centuries in oil palm plantation. Nearly four million hectares or more than one-third of the land area is used for oil palm plantation in 2003 (Yusoff and Hansen, 2007). Based on the statistics reported by the Malaysian Palm Oil Board (MPOB) recently, the land use for oil palm plantation has increased to 4.49 million hectares and Sabah contributes the largest land area for oil palm cultivation. Rapid development on palm oil plantation has given impact on economic growth in palm oil mill industries but indirectly this industry is also generating a lot of wastes such as POME. Therefore, it is an advantage to use the available resources for biohydrogen production in this study. Recently, biohydrogen production using POME was conducted. Based on the research reported, the main factor that influenced the biohydrogen production was inoculum size and the optimum inoculum size was 40% of POME sludge. The highest yield of biohydrogen achieved was 28.47 ml H_2 / g COD removed (Zainal *et al.*, 2018).

1.2 Problem Statement

Nowadays, 80% of the world energy requirement depends on fossil fuels. This ultimately will lead to a reduction in global fossil fuel resources. Besides that, the combustion of fossil fuels has affects the global environment mainly due to the discharge of pollutants such as carbon dioxide, sulfur dioxide, ash, soot and other compounds that can cause greenhouse effect. With the intention to recover the limitation of fossil fuels, also to produce cleaner and environment friendly combustion effect, hydrogen has been recommended as an alternative energy resource (Das, 2001).

Energy value per unit of weight carried out by hydrogen is higher than other resources of energies such as gasoline, methane, natural gas, diesel, ethanol, methanol, coal (anthracite) and wood (Basak and Das, 2007; Lam and Lee, 2011; Azwar *et al*., 2014). Therefore, more energy can be supplied by hydrogen for combustion per unit quantity. These properties make hydrogen an attractive alternative source for the energy system (Lam and Lee, 2011). Hydrogen is a clean energy that produces water as the only by-product (Johnston *et al*., 2005). Wastewater treatment plant is one of the resources for biogas production. The hydrogen gas will be used as fuel and fed into fuel cells to generate electricity for the plant (Lam and Lee, 2011). The biohydrogen production is important due to both environmental, since the process can improve the characteristics of agroindustrial wastewater, as well as the economics, since waste substrates can be used to generate biogas rich in hydrogen, a clean energy sources.

1.3 Objectives of Research

The aim of this research were to produce clean energy as well as to reduce wastes in the environment. The objectives of this research were:

i. To isolate and screen for biohydrogen producing bacteria from agroindustrial wastewater
ii. To identify selected biohydrogen producing bacteria and optimise the hydrogen production
iii. To determine the biohydrogen production from POME using selected bacteria

1.4 Scope of Research

This research was initiated to isolate bacteria from several types of wastewater including POME, palm oil refinery effluent, tapioca mill effluent and pineapple mill effluent prior to screening of biohydrogen producing microbes. The effects of carbon and nitrogen source concentrations towards biohydrogen production on selected bacteria were investigated. The optimum condition for biohydrogen production such as pH, temperature and inoculum size were determined. Once the optimised condition were achieved, this study was further continued on determining the potential of biohydrogen production from POME using selected bacteria.

1.5 Research Significance

Hydrogen gas is a source of renewable energy, which will not cause the greenhouse effect. So, it is safe and environment friendly to use hydrogen gas as a resource to generate electricity or energy. In this study, optimum condition for hydrogen production from bacteria will be determined. The results can be used as an input to improve wastewater treatment especially for POME in term of renewable energy generation in commercial scale. Integration of waste treatment and biogas facilities has a great prospective for industries (Lam and Lee, 2011).

The production of these clean bioenergy is in compliance with the national and international policy as well as the worldwide effort to develop alternative renewable and sustainable resource of energy. Thus, creating green and sustainable environment could be achieved and at the same time, the utilisation of waste could be the key to developing a new energy carrier for the future energy resources.

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